PB81-155574

Mutagenistic Testing of Industrial Wastes from Representative Organic Chemical Industries

East Central Oklahoma State Univ., Ada

Prepared for

Robert S. Kerr Environmental Research Lab.
Ada, OK

Jan 81

U.S. Department of Commerce
National Technical Information Service
NTIS.

TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
BEGIN FIRST LINE OF TEXT HERE
DROPPED HEAD: BEGIN SECTIONS HERE

PB81-155574

EPA-600/2-81-007
January 1981

MUTAGENISTIC TESTING OF INDUSTRIAL WASTES FROM REPRESENTATIVE ORGANIC CHEMICAL INDUSTRIES

by

Susan Stinnett, Don Noble,
Elmer Brown, and Harry Love
East Central Oklahoma State University
Ada, Oklahoma 74820

E.P.A. Grant No. R806557

Project Officer

John E. Matthews
Source Management Branch
Robert S. Kerr Environmental Research Laboratory
Ada, Oklahoma 74820

ROBERT S. KERR ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
ADA, OKLAHOMA 74820

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"
PAGE NUMBER
EPA-287 (Cin.) (4-76)

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/2-81-007	2.
3. RECIPIENT'S ACCESSION NO. PB81 155574	
4. TITLE AND SUBTITLE Mutagenistic Testing of Industrial Wastes from Representative Organic Chemical Industries	5. REPORT DATE January 1981 Issuing Date. 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Susan Stinnett, Don Noble, Elmer Brown, and Harry Love	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS East Central Oklahoma State University Ada, Oklahoma 74820	10. PROGRAM ELEMENT NO. 1BB610/C33B1B 11. CONTRACT/GRANT NO. Grant No. R806557
12. SPONSORING AGENCY NAME AND ADDRESS Robert S. Kerr Environmental Research Laboratory U.S. Environmental Protection Agency P. O. Box 1198 Ada, OK 74820	13. TYPE OF REPORT AND PERIOD COVERED Final/4-17-79 to 4-17-80 14. SPONSORING AGENCY CODE 600/15
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

The general applicability of the Ames test for screening wastewater samples was investigated. Application of the Ames test to raw and treated wastewaters from representative organic chemical industries involved the investigation of several problems: (1) the feasibility of using the Ames test to detect mutagens in wastewater samples; (2) the relative effectiveness of various waste treatment processes; (3) the mechanics of establishing an Ames testing program; and (4) the economics of using the test in routine environmental screening.

Sample results were analyzed on the basis of relative increases in revertant colonies on test plates as compared to control spontaneous reversion plates. For a sample to be scored "positive," six replicate test plates gave an average count of at least twice the control value. Of 46 samples provided, 6 were interpreted as positive, 22 were interpreted as negative, and 18 were not tested.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Water pollution Methodology Carcinogens Bioassay Mutagens	Ames test	06F

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE
6-1/2"
9-1/8"
BEGIN LAST LINE OF TEXT
3/8"
BOTTOM OF IMAGE AREA. OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
PAGE NUMBER
EPA-287 (Cin.) (4-76)

DISCLAIMER

This report has been reviewed by the Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

FOREWORD

The Environmental Protection Agency was established to coordinate administration of the major Federal programs designed to protect the quality of our environment.

An important part of the Agency's effort involves the search for information about environmental problems, management techniques and new technologies through which optimum use of the Nation's land and water resources can be assured and the threat pollution poses to the welfare of the American people can be minimized.

6-1/2"

As one of these facilities, the Robert S. Kerr Environmental Research Laboratory is responsible for the management of programs to: (a) investigate the nature, transport, fate and management of pollutants in groundwater; (b) develop and demonstrate methods for treating wastewaters with soil and other natural systems; (c) develop and demonstrate pollution control technologies for irrigation return flows; (d) develop and demonstrate pollution control technologies for animal production wastes; (e) develop and demonstrate technologies to prevent, control or abate pollution from the petroleum refining and petrochemical industries, and (f) develop and demonstrate technologies to manage pollution resulting from combinations of industrial wastewaters or industrial/municipal wastewaters.

The Ames test has been prominently mentioned as a method for determining whether a substance exhibits mutagenic characteristics. This report considers the possibility of using this procedure to determine whether complex organic chemical wastewaters exhibit mutagenic characteristics; and, if so, the relative effectiveness of various waste treatment processes in removing substances contributing these mutagenic effects.

Clinton W. Hall

Clinton W. Hall
Director
Robert S. Kerr Environmental Research Laboratory

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA; OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

ABSTRACT

The general applicability of the Ames test for screening wastewater samples was investigated. Application of the Ames test to raw and treated wastewaters from representative organic chemical industries involved the investigation of several problems: (1) the feasibility of using the Ames test to detect mutagens in wastewater samples; (2) the relative effectiveness of various waste treatment processes; (3) the mechanics of establishing an Ames testing program; and (4) the economics of using the test in routine environmental screening.

Samples were supplied in groups of influent, activated carbon-treated influent, effluent, and activated carbon-treated effluent. Each was passed through various types of glass filters to achieve sterility prior to testing. Dose levels of filtered samples ranging from 0.5 microliter to 500 microliters were incorporated into the test plates. Results were analyzed on the basis of relative increases in revertant colonies on test plates as compared to control spontaneous reversion plates. For a sample to be scored "positive", six replicate test plates gave an average count of at least twice the control value. Of 46 samples provided, 6 were interpreted as positive, 22 were interpreted as negative, and 18 were not tested.

This report was submitted in fulfillment of Grant No. R806557 by East Central University under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from April 17, 1979 to April 17, 1980, and work was completed as of December 31, 1979.

BEGIN LAST LINE OF TEXT
3/8"
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS

EFA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD, BEGIN SECTIONS HERE

CONTENTS

Foreward iii
Abstract iv
Tables vi
Abbreviations and Symbols vii
Acknowledgment viii

1. Introduction 1
2. Conclusions 2
3. Recommendations 3
4. Materials and Methods 4
   - Media 4
   - Solutions 6
   - Sample Preparation 8
   - Bacterial Test Strains 9
   - *Salmonella* Checkouts 9
5. Experimental Procedures 11
6. Results and Discussion 13
   - Potential for false positives 16
   - Potential for false negatives 17

References 18
Appendix
Average of Revertant Colonies in Ames Tests 19

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE

TABLES

Number		Page
1.	Samples Provided by EPA for Ames Testing	7
2.	Standard Mutagens for Salmonella Checks	10
3.	Dose Levels in a Typical Ames Test Procedure (In µl)	12
4.	Spontaneous Reversion Rates	14
5.	Spontaneous Reversion Rates Reported by Ames	14
6.	Samples Whose Test Counts Averaged At Twice the Average Spontaneous Reversion Rate	15
7.	Samples Showing Negative Results in Ames Testing	15
8.	Average Revertant Colonies in Ames Tests	20

6-1/2"
9-1/8"
BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE

LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

°C	-- degrees, Celsius
cm	-- centimeter
g	-- gram
µg	-- microgram
µl	-- microliter
µm	-- micrometer
µmol	-- micromole
mg	-- milligram
ml	-- milliliter
mM	-- millimolar (or millimoles per liter)
ng	-- nanogram
psig	-- pounds per square inch, gauge

SYMBOLS

HCl	-- hydrochloric acid
KCl	-- potassium chloride
K_2HPO_4	-- potassium monohydrogen phosphate
KH_2PO_4	-- potassium dihydrogen phosphate
$MgCl_2$	-- magnesium chloride
$MgSO_4$	-- magnesium sulfate
NADP	-- nicotinamide adenine dinucleotide phosphate
$NaNH_4HPO_4$	-- sodium ammonium phosphate
Na_2HPO_4	-- sodium monohydrogen phosphate
NaH_2PO_4	-- sodium dihydrogen phosphate

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-267 (Cin.) (4-76)

TYPING GUIDE SHEET
BEGIN FIRST LINE OF TEXT HERE
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

ACKNOWLEDGMENTS

For collecting and handling the samples, as well as for considerable guidance and technical assistance, we wish to thank John Matthews, Project Officer, and James McNabb, Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency. We also appreciate the assistance of Dr. James Vaughn, Brookhaven National Laboratory; Dr. Bruce Ames and Ms. Dorothy Maron, Department of Biochemistry, University of California, Berkeley.

For unprecedented kindness and competence, we acknowledge Van May, Cynthia Norman, Pat Mason, and Mike Mason.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA. OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.) (4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

SECTION 1

INTRODUCTION

An increasing concern with the possible introduction of carcinogens into the environment has resulted in a search for a simple, sensitive, and reliable method for the detection of these chemicals. One such method has achieved considerable attention among industries and governmental agencies interested in routine screening for potential carcinogens in water supplies. The Ames test, developed by Bruce Ames, University of California, Berkeley, is a simple and relatively inexpensive test currently in use for this purpose.

The Ames test was originally designed to determine the ability of a specific compound to cause mutations. As most carcinogens are also mutagens, a correlation has been made between positive results in the test and potential carcinogenicity. Test strains of bacteria, supplemented with extracts of rat liver to simulate mammalian metabolism, respond readily to the presence of most mutagens in minute quantities. The test is currently being investigated as a possible method for detecting mutagens in industrial effluents.

In conducting this study, the general applicability of the Ames test to screening wastewater samples was investigated. The problems involved in preparing samples for testing and in interpreting the results are markedly different for environmental samples as opposed to pure compounds or extracts. In applying the test to raw and treated industrial wastewaters, several objectives were examined: (1) the determination of the feasibility of using the Ames test to detect carcinogens in the environment; (2) the relative effectiveness of various waste treatment procedures; (3) the mechanics of establishing an Ames testing program; and (4) the economics of using the test for screening. The controversial correlation between mutagenicity and carcinogenicity was beyond the scope of this study.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

SECTION 2

CONCLUSION

On the basis of our experience and the data observed, we present the following conclusions:

(1) It is possible to detect positive mutagenesis in at least some samples with the procedure used in this project. Because the mutagenic agents are so dilute in wastewater, negative results obtained from these tests do not necessarily indicate the absence of mutagens.

(2) There is a potential for obtaining false positives using this procedure.

(3) The effectiveness of waste treatment cannot fully be determined in a screening program. That determination warrants a separate study.

(4) While the test does not appear to require much sophistication, it requires a large laboratory used exclusively for Ames testing with a large capacity automatic autoclave, considerable refrigerator space, and a laminar-flow hood designed for use with carcinogens. The laboratory will have a very large demand placed on its electrical circuits.

(5) Preparation for an Ames test screening project requires a minimum of 6 to 12 months. Some of the larger pieces of equipment needed are not manufactured routinely, and there may be an unavoidable delay of several months after the bids are accepted while a freezer or incubator is being manufactured. To minimize this problem, equipment and supplies should be ordered well in advance of the date Ames testing is scheduled to commence. Other unavoidable delays will occur, as biochemicals such as nicotinamide adenine dinucleotide phosphate (NADP) and S9 liver homogenate are often back-ordered. As unforeseen events will arise during the course of a screening project, it is impossible to determine in advance the exact amounts of such materials that will be consumed over a 6-12 month period, so they will have to be reordered, perhaps several times.

(6) The initial expense of establishing a laboratory for screening samples by Ames testing will be high. Once the laboratory is equipped and staffed with people who have had time to perfect the necessary technique, routine testing will be relatively inexpensive when compared to animal studies.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

SECTION 3

RECOMMENDATIONS

For each of the major goals of this project, we offer the following recommendations for careful consideration:

(1) The wastewater samples provided for this study were carefully tested according to Ames' procedures [1] and EPA's quality control guidelines [2]. The EPA should consider using the Ames test as only one of several screening tests [3]. Before an industry is judged by EPA to release either negative or positive effluents, a more thorough test should be applied; including basic Ames testing replicated several times for each dose level and including a wider range of dose levels.

(2) Sample preparation should be more thoroughly investigated. After repeated filtering, bacteria remained in some samples. Although they did not grow on minimal media, other samples might contain small organisms that could grow on minimal media and ruin the tests. A reliable filtration technique needs to be developed.

(3) To answer the statement that some mutagens might have been adsorbed onto particulate matter that was removed by filtration, the contents trapped by these filters should also be analyzed. Some physical means of sterilization such as radiation or sonication might be employed to advantage here, either along with or in place of filtration.

(4) When considering extensive Ames testing by small laboratories, existing physical plants should be carefully observed. We recommend that minimum equipment used for this type of screening should include 4 or 5 large capacity heat blocks to be used in place of water baths, a Revco freezer, a laminar-flow hood designed for work with carcinogens, two incubators used only for Ames testing, and an automatic electronic colony counter. Without this equipment it is very difficult to maintain a screening program that tests up to 10 waste samples per week.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/4"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

SECTION 4

MATERIALS AND METHODS

MEDIA

Four media are required for Ames testing: Vogel-Bonner minimal medium E (VB), top agar supplemented with traces of histidine and biotin (TA), nutrient agar + 0.5% NaCl (nutrient agar or NA), and nutrient broth + 0.5% NaCl (nutrient broth or NB). Nutrient agar and broth frequently contain materials that alter the growth and spontaneous reversion of the tester strains of Salmonella. This problem was experienced midway through this study. Dorothy Maron of Ames' laboratory at Berkeley suggested that Oxoid medium #2 be substituted for nutrient media. This substitution was not made in this project since it would have nullified data already generated and insufficient sample volumes were available to rerun all tests already completed. Therefore, all data in this report were collected with nutrient media; however, subsequent Ames testing should use Oxoid #2 from the beginning. Oxoid medium #2 may be obtained from K. C. Biological, P. O. Box 5441, Lenexa, Kansas, 66215.

The detailed work plan approved for this study included a time period of 3-4 weeks during which all media and solutions were to have been prepared and stored for later use. This proved impossible for several reasons outlined below.

VB medium consists of a salt solution to which agar and glucose are added [4]. To prepare VB, a stock salt solution (5x strength) is first prepared by dissolving in distilled water: 1.0 g magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$), 10.0 g citric acid ($H_3C_6H_5O_7 \cdot H_2O$), 50.0 g potassium monohydrogen phosphate (K_2HPO_4), and 17.5 g sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4\ H_2O$). The salts are added individually to a quantity of distilled water in a 1000-ml volumetric flask, and they must be added in the order given, each one being dissolved completely before the next is added. This prevents precipitation of salt complexes which cannot be redissolved. The solution is brought to a final volume of one liter by adding distilled water to the mark. This stock 5x salt solution may be stored at 4°C for several days. When the actual working medium is made, 200 ml of this stock solution were mixed with 15 g of agar and 300 ml of distilled water. This mixture was autoclaved at 121°C for 20 minutes. It was held at 45°C until use, at which time 500 ml of warm distilled water in which 20 g of glucose had been autoclaved were added to the salt solution. The glucose solution has to be autoclaved separately to prevent toxic complexes (inhibitory to bacterial growth) from being formed.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

The agar-salt solution can only be melted once, during sterilization, and cannot be held at 45°C for even a few hours because the large concentration of citrate chars very easily and the medium darkens. Remelting solid VB is not acceptable, so it is impossible to prepare it early and store it in large, undispensed volumes as had been planned. Since each Ames test requires approximately 100 plates of VB and each Salmonella check uses approximately 75 plates, the total number of VB plates used in this project was well above the refrigerator capacity of the laboratory. As even brief storage at 4°C produces condensate on the plates, it was more satisfactory to prepare enough VB plates for 3 or 4 days testing (approximately 1000 plates) and store them at room temperature for several days before use. This allowed the normal condensation to dissipate somewhat and also allowed contaminated VB plates to be discarded before use.

TA was to have been prepared, dispensed in tubes, autoclaved, then stored at 4°C and remelted immediately prior to use. This technique yielded control plates with no revertant colonies early in the testing. Although no literature reference was found to explain the phenomenon, it appeared that the histidine-biotin solution was heat-labile. Personal communications with others involved in Ames testing supported this conclusion (James Vaughn, Brookhaven National Laboratory, New York; Bill Stang, EPA, Denver; Dorothy Maron, Univ. of Calif., Berkeley). TA was then prepared by dissolving 3.35 g of Difco agar and 2.5 g of NaCl in distilled water to make 500 ml, autoclaving, and storing at 4°C until use. The histidine-biotin supplement, containing 0.1048 g of L-histidine and 0.1221 g of biotin in distilled water to make 1000 ml, was filtered through a 0.45 micrometer (μm) Millipore membrane filter into a sterile filter flask. It was then aseptically dispensed into four sterile 250-ml Erlenmeyer flasks and refrigerated. On the day of TA preparation, a 50-ml portion of the histidine-biotin solution was refiltered through a Gelman acrodisc filter assembly (0.45 um) into a large, sterile culture tube and was allowed to warm to room temperature. A 500-ml portion of the agar solution was melted and cooled to 45°C in a water bath. A 50-ml portion of warm sterile histidine-biotin solution was aseptically added to the melted agar solution and swirled carefully to mix. Using a sterile 5-ml Cornwall pipettor, the TA was then aseptically dispensed into pre-sterilized tubes in 2-ml volumes. To keep contamination from entering the TA, the tip of the pipettor was quickly flamed periodically. This made it necessary to replace the valve assembly more frequently than usual.

TA dispensing was done in a large 45°C water bath, as cooling below 45°C quickly solidifies TA and remelting inactivates the histidine-biotin supplement. The water level had to be carefully regulated at just above the depth of TA in the tubes; too much water tended to increase the contamination problem, and too little water cooled the top agar so it solidified. Covers over the baths allowed condensation to fall on the caps of the tubes and caused contamination problems, so they were removed. This increased the frequency of monitoring the bath levels and temperatures to four or five checks per day, one of which was early in the morning and one late in the evening.

Maintaining asepsis throughout TA preparation was the most difficult technique to control, and some data compiled very early in the testing

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE

schedule had to be discarded because of TA contamination. Screw-capped TA tubes suggested by Ames were abandoned after a one-week trial because manipulating the caps proved too difficult to allow aseptic conditions to be maintained. As each test represented considerable time, effort, and money, subsequent batches of top agar were prepared no less than 24 hours prior to use. After dispensing, each batch was tested for sterility by randomly selecting several tubes, pouring each over a NA plate, and incubating overnight at 37°C. This allowed contaminated batches to be identified and discarded before use. Once the procedure was perfected, a responsible student assistant who was proficient in microbiological techniques was able to perform it routinely after several practice runs.

SOLUTIONS

S9 fraction of rat liver homogenate prepared from Aroclor-induced rats [1] was obtained in 2-ml and 5-ml vials from Litton Bionetics, Kensington, Maryland. It arrived frozen, packed in dry ice, and was immediately transferred to a Revco freezer for storage at -80°C.

S9 mix was prepared early in the morning of each day of testing. The procedure outlined below was used for 5-ml vials of S9; it was cut proportionally for 2-ml vials. A 5-ml vial of S9 allowed sufficient mix for three Ames test.

S9 mix was prepared so that each ml of the final solution contained 0.07 ml of S9, 8 micromole (μmol) of magnesium chloride ($MgCl_2$), 33 μmol of potassium chloride (KCl), 5 μmol of glucose-6-phosphate, 4 μmol of NADP, and 100 μmol of sodium phosphate buffer (pH 7.4). Stock salt solutions of 22.856 millimolar (mM) $MgCl_2$ (1.1616 g $MgCl_2 \cdot 6\ H_2O$/ 250 ml distilled water), 128.562 mM KCl (2.3763 g KCl/250 ml), 246.93 mM NaH_2PO_4 (8.518 g NaH_2PO_4/ 250 ml), and 246.93 mM sodium monohydrogen phosphate (Na_2HPO_4) (33.098 g Na_2HPO_4/250 ml) were prepared and stored at 3-5°C. A buffer solution (pH 7.4) was prepared fresh each week by mixing appropriate amounts of the sodium phosphate salt solutions. The buffer solution was stored in the refrigerator for one week.

On the morning of the day that S9 mix was needed, a buffered solution was prepared by mixing 25 ml of $MgCl_2$ stock solution, 12.5 ml of KCl stock, 28.93 ml of sodium phosphate buffer, 100.7 mg of glucose-6-phosphate (monosodium salt) and 229 mg of NADP (sodium dihydrate). This solution was filtered through a 0.45 μm pore Gelman acrodisc disposable filter assembly into a sterile 250-ml Erlenmeyer flask and placed on ice. A 5-ml vial of frozen S9 was hand-warmed on the outside until it could be poured into the flask. The S9 was allowed to finish thawing in the flask, after which the flask was gently swirled to thoroughly mix the contents. The S9 mix was kept on ice throughout the 1-day testing procedures. Any excess was discarded at the end of the working day.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.) (4-76)

TABLE 1. SAMPLES PROVIDED BY EPA FOR AMES TESTING

Sample No.	Industrial Category	Sample Point	Sample No.	Industrial Category	Sample Point
1*	Petrol. Ref.	Inf. C-T	25*	Pesticide	Inf. C-T
2	"	Inf.	26	"	Inf.
3*	"	Eff. C-T	29*	Pharm.	Inf. C-T
4	"	Eff.	30	"	Inf.
5	Org. & Plas.	Inf. C-T	31*	"	Eff. C-T
6	"	Inf.	32	"	Eff.
7	"	Eff. C-T	33*	Wood Pres.	Inf. C-T
8	"	Eff.	34	"	Inf.
9*	Pesticide	Inf. C-T	35*	"	Eff. C-T
10	"	Inf.	36	"	Eff.
11	"	Eff. C-T	37*	Org. & Plas.	Inf. C-T
12	"	Eff.	38	"	Inf.
13*	Rubber	Inf. C-T	39	"	Eff. C-T
14	"	Inf.	40	"	Eff.
15*	"	Eff. C-T	41*	Wood Pres.	Inf. C-T
16	"	Eff.	42	"	Inf.
17*	"	Inf. C-T	43*	"	Eff. C-T
18	"	Inf.	44*	"	Eff.
19*	"	Eff. C-T	47*	"	Eff.
20	"	Eff.	48	"	Eff.
21*	Pharm.	Inf. C-T	49*	Pharm.	Inf. C-T
22	"	Inf.	50	"	Inf.
			53	Org. & Plas.	Eff. C-T
			54	"	Eff.

* No test conducted on these samples.

Abbreviations:

Petrol. Ref. - Petroleum refinery
Org. & Plas. - Organics & Plastics
Pharm. - Pharmaceuticals
Wood Pres. - Wood preservatives

Inf. C-T - Influent, carbon-treated
Inf. - Influent
Eff. C-T - Effluent, carbon-treated
Eff. - Effluent

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

SAMPLE PREPARATION

Samples collected by the Robert S. Kerr Environmental Research Laboratory, Ada, Oklahoma, were pre-filtered through glass wool before transfer to the investigators. A 30-ml volume of sample, numbered and designated influent, carbon-treated influent, effluent, or carbon-treated effluent (Table 1) was supplied for each of 46 different wastewaters. These were collected from 14 industrial sites involved in petroleum refining or production of organic chemicals, pesticides, wood preservatives, rubber, or pharmaceutical products. Samples were kept at 3-5°C throughout the grant period.

One-ml aliquots of each sample were initially tested for sterility by spreading on NA plates. All were heavily contaminated with bacteria, making Ames testing impossible. The usual membrane-filtration technique using acetate filters was inappropriate for these samples, as the organic compounds they contained might have reacted with or dissolved the filters. As an alternative, Bush bacteriological fritted glass filters, pore diameter 0.9-1.4 μm (Corning #33992) were purchased from Sargent-Welch. The filters were washed in dilute hydrochloric acid (HCl), rinsed in distilled water, wrapped in heavy aluminum foil and sterilized in an oven at 260°C overnight before use. Samples were filtered under pressure of 5-15 psig. The compressed air was supplied by an Ametrol portable air tank equipped with a hand pump and regulator.

Because many of the samples required several hours to pass through the Bush filters (some required 48 hours or more), another alternative filtration system was investigated. Gelman glass membrane filters, type A/E (pore size 0.45 μm) were used with a Millipore filtration apparatus to collect the samples in sterile filter flasks. This technique failed to give sterile samples, and because of limited time available for testing, it was abandoned without further study.

After filtration, the samples were stored at 3-5°C. Each was checked for sterility by plating 0.1-ml aliquots on two NA plates and two VB-TA plates. After incubation for 24 hours at 37°C, most samples appeared to be sterile. However, after 48 hours at 37°C, several showed heavy contamination with micro-organisms that produced minute white colonies on NA and failed to grow on VB-TA plates. These samples were refiltered, sometimes several times, until the NA plates were negative after 48 hours.

Occasionally there was a delay of several days between sterility certification and Ames testing. In some samples the sample sterility controls done with the Ames test showed contamination with the same type of colonies. It appears that these samples were not actually sterilized, as a few cells passed through the filter (too few to be caught by the checking procedure). Those cells apparently reproduced in the refrigerated samples. In some of these cases, the tests were repeated on freshly-certified samples, and no significant difference appeared in the data. Subsequent data were used for all tests in which the VB-TA sterility controls were sterile.

BEGIN LAST LINE OF TEXT HERE
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-2F7 (Cin.) (4-76)

BACTERIAL TEST STRAINS

Four cultures of Salmonella typhimurium histidine auxotrophs, designated TA 98, TA 100, TA 1535, and TA 1537 [1] were supplied by the laboratory of Bruce Ames, Department of Biochemistry, University of California, Berkeley. All four strains require histidine and biotin supplements for growth on minimal media and are sensitive to ultraviolet irradiation and crystal violet. TA 98 and TA 100 also carry a plasmid imparting resistance to ampicillin, an antibiotic that kills the other strains.

Lyophilized cultures supplied by Ames were initially streaked on plates of nutrient agar and were cultured in nutrient broth. Frozen permanent reserve and working stock cultures were prepared by mixing 2 ml of the initial broth bulture with 0.1 ml of spectrophotometric grade dimethylsulfoxide (DMSO) in sterile glass vials. Each vial was immersed in acetone and dry ice for quick freezing. The frozen permanent reserves were maintained at -80°C in a Revco freezer, model ULT-185-AHG.

To avoid damage to the frozen working stocks, Ames suggested minimizing thawing and refreezing by preparing stock NA plates once a month from the frozen reserves. These plates, kept at 4°C in a refrigerator, served as the source of inoculum for each set of working broth cultures.

Once each week, six 10-ml nutrient broth cultures and one NA slant culture were inoculated from the stock plate for each strain. Broth cultures were incubated overnight in a shaking 37°C water bath, and NA slant cultures were incubated overnight at 37°C in a Precision Scientific incubator. Broth culture cell densities approximated 10^9 cells per ml as determined by comparing spontaneous reversion rates with those of 10^9 cells per ml cultures from Ames' laboratory [1; personal communication, Dorothy Maron, 8-20-79]. One broth culture of each strain was randomly chosen for checkout procedures. The other five were refrigerated and used later for testing.

SALMONELLA CHECKOUTS

Each randomly chosen broth culture was streaked on VB medium. Histidine and biotin requirements were determined by streaking each strain on a VB plate spread with 0.1 ml of 0.1 M histidine and on a VB plate spread with 0.1 ml of 0.5 mM biotin. When streaked directly from nutrient broth, enough biotin was transferred from the broth to allow a few colonies to grow on the histidine-supplemented plates. This was eliminated by making distilled water suspensions of cells grown on NA slants and using these suspensions as sources of inoculum for the VB-histidine-biotin checks. All tests described below were performed on the broth cultures.

Sensitivity to ampicillin or crystal violet was determined by spreading 0.1 ml of culture on each of two NA plates. A filter paper disc impregnated with either 10 micrograms (μg) crystal violet or 10 μg ampicillin was placed in the center of each plate. Sensitivity was indicated by a clear zone of inhibition around the disc following a 48-hour incubation at 37°C.

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

Ultraviolet sensitivity was determined by spreading 0.1 ml of culture on two NA plates and exposing one-half of each plate to a germicidal (254 nm) Mineralight UV lamp, 110 volts and 0.16 amps. One plate for each strain was exposed at a distance of 33 cm, the other at 19 cm. TA 98 and TA 100 were exposed for 8 seconds, while TA 1535 and TA 1537 were exposed for 6 seconds. After 48 hours at 37°C, sensitivity was determined by observing significantly reduced or no growth on the irradiated side of the plate.

Positive mutagenesis was determined by plating 0.1 ml of culture in TA (with histidine and biotin supplement), with or without 0.5 ml of S9 mix, and spot testing with the suitable mutagen (Table 2). TA 1537 was very difficult to mutate, and best results were obtained by incorporating the mutagens into the TA before pouring. All work with mutagens was done wearing gloves and protective clothing, in a hood vented to the outside. Plates containing mutagens were incubated in an outdoor-vented 37°C Precision Scientific incubator, counted under the hood, disinfected with amphyl, and placed in bio-hazard bags for disposal (see section on waste disposal). Mutagen solutions were purchased from Nanogens Inc., at concentrations of 100 ng/µl. DMSO was the solvent used.

Spontaneous reversion rates for each strain were determined with and without S9. Three to five plates of each strain were prepared by adding 0.1 ml of the overnight broth culture to TA and pouring over VB. An equal number of plates containing 0.1 ml of culture and 0.5 ml of S9 mix were prepared at the same time. These were incubated for 48 hours at 37°C.

TABLE 2. STANDARD MUTAGENS FOR SALMONELLA CHECKS

Strain	Mutagen Used	Concentration	S9 Requirement
TA 98	2-aminofluorine	100 µl	+
TA 100	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	25 µl	-
TA 1535	MNNG	25 µl	-
TA 1537	MNNG	25 µl	-
	4-aminobiphenyl	100 µl	+

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

SECTION 5

EXPERIMENTAL PROCEDURES

The procedures used in this project were modifications of the techniques of Ames [1] and the quality control guidelines of the EPA [2]. It was essential to modify the techniques because pure compounds were not used and there was a minimum amount of time for testing.

Each sample was prefiltered, checked for sterility, then refrigerated. To minimize evaporation of volatile organics from the samples, plastic tape was used to seal the containers. Each sample was diluted 1:20 by pipetting 5.0 ml of sample into a sterile 100-ml volumetric flask and diluting to the mark with sterile distilled water. Sterility controls were done on the unused dilution water, the diluted sample, and the concentrated sample immediately following the dilution process. The diluted and undiluted samples were also checked for sterility during and after the testing. Each sterility check was done by plating 0.1 ml of sample on NA and 0.1 ml in TA on VB, then incubating at 37°C for 48 hours.

A set of TA tubes for checking sterility of dilution water, samples and agar was kept in the 45°C water bath along with a group of 80 TA tubes for Ames testing. These were arranged in four sets of 20 tubes, and were dosed as shown in Table 3. Four tubes of each set received 10 µl of the 1:20 dilution, a final dose level of 0.5 µl. Four tubes of each set received 100 µl of the 1:20 dilution, a final dose level of 5 µl. Four tubes of each set received 20 µl of the undiluted sample, four received 100 µl of undiluted sample, and the last four in each set received 500 µl of undiluted sample. No more than 500 µl could be used for two reasons: a small volume of sample was provided, and larger volumes when added to 0.5 ml S9 mix would have diluted the top agar so it could not solidify. The latter was verified by testing with water blanks instead of sample. All doses were delivered with Oxford pipettors using sterile tips.

Following the dose additions, the test was begun with set one containing 20 tubes. Ten of these (in the column on the right in set #1, Table 3) were seeded with 0.1 ml each of a NB culture of TA 98, which had been previously checked for genetic stability. Each tube was then mixed with a vortex mixer and poured over a VB plate. The plates were immediately placed in a dark box to prevent photorepair mechanisms from interfering with the detection of mutations. When the top agar solidified, they were inverted and incubated at 37°C. The second group of ten tubes in set #1 was then seeded as before with TA 98. Before each tube was mixed, 0.5 ml of S9 mix was added. Within 20 seconds of the addition of S9 to a tube, it was mixed, poured, and placed in

BEGIN LAST LINE OF TEXT HERE
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cln.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD BEGIN SECTIONS HERE

the dark. Speed was essential to prevent inactivation of the enzymes at 45°C.

The above procedure was then repeated, substituting TA 100 in set #2, TA 1535 in set #3, and TA 1537 in set #4. All plates were inverted and incubated at 37°C for 48 hours, after which they were counted using a mechanical register and a Quebec colony counter.

To complete a test, an experienced worker needs 2.5 hours. A worker recording data needs 2-3 hours to count and record the data from one test. As the data were recorded, the plates were autoclaved and discarded.

TABLE 3. DOSE LEVELS IN A TYPICAL AMES TEST PROCEDURE (IN μl)

Set #1: TA 98		Set #2: TA 100		Set #3: TA 1535		Set #4: TA 1537	
S9	no S9	S9	no S9	S9	no S9	S9	no S9
500	500	500	500	500	500	500	500
500	500	500	500	500	500	500	500
100	100	100	100	100	100	100	100
100	100	100	100	100	100	100	100
20	20	20	20	20	20	20	20
20	20	20	20	20	20	20	20
5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE

SECTION 6

RESULTS AND DISCUSSION

The Ames test was designed to determine whether a pure compound is able to cause bacteria to mutate. The significance of this rather simple test system lies in the correlation between the ability to cause bacterial mutations and the ability to cause mutations in mammals, including humans [1]. Testing chemicals on animals is an expensive and time-consuming process. It is hoped that the much simpler Ames test can give valid preliminary information about mutagens. Our results suggest that the test may be useful as one tool for the preliminary screening of wastewaters for potential mutagenic activity.

The test works by inducing mutations in the areas of the bacterial chromosome that have already been mutated. To appear on VB-TA plates, the histidine-biotin deficient mutants must regain the ability to synthesize these compounds. This is accomplished when the mutagenic compound reacts with the genetic material, reversing the original mutations. Since this phenomenon occurs with some regularity in the absence of mutagens, the background "spontaneous reversion" rate must be determined before and during Ames testing.

It is generally accepted [1,3] that a given amount of material is considered to have mutagenic activity if test plates incorporating that dose show at least twice as many colonies as the average number of spontaneous revertants for the bacteria used. Data compiled for spontaneous reversions are presented in Table 4. Three of the four strains showed lower rates than expected from Ames' data as summarized in Table 5. This is probably the result of the media used.

A summary of the positive results obtained during this project appears in Table 6. Complete data appear in the appendix. We initially tested each influent and effluent using the procedure outlined previously. In each test, we scored as "positive" plates of TA 98 with 46 or more colonies, plates of TA 100 with 224 or more colonies, plates of TA 1535 with 22 or more colonies, and plates of TA 1537 with 20 or more colonies. All other plates were scored as "negative". If an influent or an effluent showed a positive score at any dose level for any of the test strains, corresponding dose levels of the carbon-treated samples were tested. When an influent or an effluent showed no positive scores, the corresponding carbon-treated samples were not tested. Tests scored as positive were repeated; instead of averaging two plates at the positive dose level, six plates were averaged. Only those dose levels that scored positive as the average of six test plates appear in

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Clin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE
6-1/2"
9-1/8"

TABLE 4. SPONTANEOUS REVERSION RATES

Culture	No. of tests	$\bar{x}$	Range
TA 98	29	23	10-40
TA 100	30	114	86-192
TA 1535	34	11	8-16
TA 1537	31	10	4-18

TABLE 5. SPONTANEOUS REVERSION RATES REPORTED BY AMES [1]

Culture	$\bar{x}$	Range
TA 98	40	30-50
TA 100	160	120-200
TA 1535	20	10-35
TA 1537	7	3-15

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA; OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cln.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD BEGIN SECTIONS HERE

TABLE 6. SAMPLES WHOSE TEST COUNTS AVERAGED AT LEAST TWICE THE AVERAGE SPONTANEOUS REVERSION RATE

Sample No.	Dose Level	Affected Test Strain	S9 Requirement
6 (Infl.)	5 µl	TA 100	+
	100 µl	TA 100	-
8 (Effl.)	5 µl	TA 100	+
18 (Infl.)	500 µl	TA 100	-
40 (Effl.)	500 µl	TA 1535	+
42 (Infl.)	5 µl	TA 100	-
	25 µl	TA 100	-
	100 µl	TA 100	-
50 (Infl.)	100 µl	TA 100	+
	500 µl	TA 100	-

TABLE 7. SAMPLES SHOWING NEGATIVE RESULTS IN AMES TESTING

Sample No.	Sample No.
2	22
4	26
5	30
7	32
10	34
11	36
12	38
14	39
16	54
20	

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA; OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-75)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

Table 6. Table 7 lists the samples that yielded negative scores.

Positive results were anticipated because some of the molecules generated by organic chemical industries have shown positive results in pure compound testing. In industrial wastewaters, however, the exact chemicals present and their individual concentrations are unknown. It is probable that some mutagens may be present in a sample, but in concentrations below the threshold of detection using these methods. Therefore, it is remarkable that positive results were obtained in this study.

Some workers attempting to use the Ames test to screen wastewaters have approached the concentration problem by extracting the samples with methylene chloride [personal communication, Bill Stang, EPA, Denver]. We chose not to do this for two reasons: methylene chloride is a known carcinogen, and handling it increases the hazard and the time needed in sample preparation; also, because it is a mutagen, any residue of methylene chloride remaining in the extracts could alter the results of the testing. By eliminating the extraction procedure, we have eliminated these objections while raising others. The lack of concentration in our samples may lead to negative scores that might be positive if extracts were tested. Also, it is obvious that we are testing the soluble phase of the samples; any mutagens in or on particulate matter have been removed prior to testing.

In reviewing the validity of using the Ames test as a screening procedure, it is important to recognize that a potential exists for obtaining false results, either negative or positive. The Ames test is a much more complex bioassay than it appears; with such a large number of controls being necessary, even the rigid maintenance of quality control standards allows a margin of error to remain.

POTENTIAL FOR FALSE POSITIVES

(1) If the spontaneous reversion rate of a group of Salmonella cultures is abnormally high, the data obtained in tests using those cultures will also be high. When compared to the average spontaneous reversion rate, the data may be high enough to receive a positive score, although the sample may not be a true mutagen.

(2) Contamination of either the Salmonella culture or the sample could yield abnormally high results. (It is probable, but not definite, that contamination would be detectable and the data would be discarded.)

(3) An abnormally high population density in one culture could produce abnormally high results.

Any of these possibilities should produce the same effect in all tubes of a test, regardless of the dose level. In this project, we saw no consistency of high results in all tubes of any test. We therefore conclude that our positive results were not caused by these factors.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cont.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD BEGIN SECTIONS HERE

POTENTIAL FOR FALSE NEGATIVES

(1) Mutagenic compounds usually exhibit a dose response curve in pure compound tests. This means that below and above certain concentrations, their mutagenic effect is not observed. It is quite probable that mutagens may be present in wastewaters even though their concentrations are too small to be detected in the assay.

(2) Many compounds require activation by mammalian enzymes before being converted to a mutagenic state. It has been determined by McCann, et. al. [5] that the type of compound used to induce the rat liver enzyme production is very significant in detecting mutagens. Some compounds in wastewater might show negative results with Aroclor-induced S9, but positive results with phenobarbitol-induced S9. Others might require induction by as yet untried compounds.

(3) The amount of S9 used in the test system affects the results. Some compounds lose their mutagenic effect in the presence of excessive S9, although they require it for activation. Others require more than the usual amount to show positive results [5]. This greatly complicates the problem of interpreting data.

There is not enough evidence to indicate that one waste treatment system sampled is more effective than another in removing potential mutagens from wastewaters, although we found no positive results from carbon-treated samples.

Because the potential for false positives is augmented by experimental error in pipetting Salmonella into TA, and because the dose levels are so unusual, the positive results obtained in sample #6 and sample #8 (Table 6) are suspect. Five µl of sample is an extremely small volume to register positive. Of all the samples tested, #42 seems to be most consistent.

The potentials for false positive or negative results are always present in this type of bioassay; they cannot be totally eliminated. Any attempt at minimizing these potentials will detract from the value of the test as a rapid screening tool, but will certainly increase the confidence in the data. It is apparent that the experimental design used in this project was suitable only for preliminary screening of samples. It is not reasonable to classify a sample as mutagenic or not mutagenic on the basis of this test alone.

Alternative bioassays, similar to the Ames test, have been developed for detecting mutagenic activity of pure compounds. These assays use yeasts, other bacteria, or tissue culture cells as the test organisms. If the Ames testing procedure outlined in this project is coupled with one or more of these assays, it may prove to be very useful as a screening technique. Ames testing should not be used as the only screening test for determining mutagenic activity of wastewaters.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD BEGIN SECTIONS HERE

REFERENCES

1. Ames, B.N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutagen Research, 31 (1975): 347-364, 1975.

2. Tracor Jitco, Inc. Quality Assurance Guidelines for Biological Testing. EPA-600/4-78-043, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Las Vegas, Nevada, 1975. 474 pp.

3. Brusick, D.J. Observations and Recommendations Regarding Routine Use of Bacterial Mutagenesis Assays as Indicators of Potential Chemical Carcinogens. In: Strategies for Short-Term Testing for Mutagens/Carcinogens, B.E. Butterworth, ed. CRC Press, West Palm Beach, Florida, 1979. pp. 3-12.

4. Vogel, H.J. and D.M. Bonner. Acetylornithase of Escherichia coli: Partial Purification and Some Properties. J. Biol. Chem. 218 (1956): 97-106, 1956.

5. McCann, J., E. Choi, E. Yamasaki, and B.N. Ames. Detection of Carcinogens as Mutagens in the Salmonella/Microsome Test: Assay of 300 Chemicals, Proc. Nat. Acad. Sci. U.S.A. 72: 5135-5139, 1975.

6. Weinstein, D. and T.M. Lewinson. A Statistical Treatment of the Ames Mutagenicity Assay. Mutation Research 51 (1978): 433-434, 1978.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

PAGE NUMBER
EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD: BEGIN SECTIONS HERE

APPENDIX

AVERAGE OF REVERTANT COLONIES IN AMES TESTS

The data contained in Table 8 represent a summary of the raw data collected during this project. Each value listed represents the mean value for all plates tested. Unless otherwise noted, 2 plates were averaged to obtain the mean. One asterisk (*) indicates an average of 4 plates, and two asterisks (**) indicate an average of 6 plates.

The sample number designates the wastewater being tested. Dose level in µl designates the amount of wastewater to which bacteria were exposed. Each test strain received each dose level without and with the addition of S9.

To interpret the data, proceed as follows:

When 0.5 µl of sample #2 was added to each of two tubes with approximately 10^8 cells of TA 98 per tube, the mixtures (when plated on VB) yielded an average of 17 revertant colonies per plate. When compared with the spontaneous reversion rates appearing in Table 4, pg. 14, this shows less than twice the average spontaneous reversion rate. When 0.5 µl of sample #2 was added to TA 98 and S9, the duplicate plates averaged 32 revertant colonies per plate. This procedure is to be followed throughout the reading of the table.

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA; OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD, BEGIN SECTIONS HERE

TABLE 8. AVERAGE OF REVERTANT COLONIES IN AMES TESTS

Sample No.	Dose in µl	98 no S9	98 S9	100 no S9	100 S9	1535 no S9	1535 S9	1537 no S9	1537 S9
2 Infl.	0.5	17	32	107	133	12	11	3	6
	5	16	27	109	148	15	13	8	17
	25	18	37	105	134	11	15	5	13
	100	17	27	107	130	13	12	5	11
	500	19	30	122	114	9	16	5	9
4 Efl.	0.5	23	30	103	159	18*	10	11	18
	5	11	31	110	141	12	12	10	11
	25	7	33	97	153	14	15	7	11
	100	11	32	125	166	11	12	11	10
	500	13	26	155	134	12	14	9	13
5 Infl. C-T	5			70	70				
	100			90*					
6 Infl.	0.5	17	37	169**	173*	11*	5**	7	8
	5	20	44	181*	230**	11*	7**	7	8
	25	14	47	166**	167*	10*	12**	7	11
	100	12	40	192**	94*	12*	8**	5	8
	500	12	29	181**	91*	17**	13**	8	12
7 Efl. C-T	0.5	20	44	134	207*	10	11	10	11
	5	15	27	152	217	6	12	3	10
	25	17	30	116	117	5	5	6	9
	100	12	18	138	109*	9	12	8	7
	500	12	32	123	119	13	9	6	6
8 Efl.	0.5	10	31*	165*	176*	15	16	11	15
	5	13	29*	182*	222**	13	12	8	10
	25	9	25*	170*	195*	16	12	11	8
	100	7	24*	172*	185*	19*	17	8	9
	500	11	27*	178*	160*	12	12	10	12

(continued)

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
6-1/2"
1/8"
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

TABLE 8 (continued)

Sample No.	Dose in µl	98 no S9	98 S9	100 no S9	100 S9	1535 no S9	1535 S9	1537 no S9	1537 S9
10 Infl.	0.5	20	41	129	134	10	11	6	10
	5	19	12	130	128	15	13	9	11
	25	25	33	168**	225**	14	18	5	11
	100	19	36	183**	214**	16*	13	6	10
	500	24	39	167**	193*	15*	14	4	10
11 Effl. C-T	0.5	14	36	168**	181**	16	16*	4	13
	5	12	48	206**	206**	11	9	6	13
	25	13	36	214**	200**	11	16	5	14
	100	19	37	166**	185*	9	16	10	8
	500	12	35	178*	181	14	14	9	9
12 Effl.	0.5	30	33	176*	158*	16	15	12	12
	5	22	34	183*	154*	10	10	12	12
	25	10	50	162**	161*	10	11	13	8
	100	14	42	154**	172*	7	15	2	10
	500	17	41	167*	160*	18	12	12	19
14 Infl.	0.5	17*	47	68*	163*	16	7	13	13
	5	18	36	172*	213*	14*	10	8	7
	25	29	39*	118	203*	8	10	9	9
	100	33	44**	146	245*	7	13	8	9
	500	23*	33	196	248*	11	12	13	7
16 Effl.	0.5	10	28	136	127	10	15*	10	21
	5	14	28	100	135	13	9	6	5
	25	7	38	133	131	11	10	5	8
	100	16	26	152	136	12	14	12	8
	500	7	30	118	127	16	8	31	6
18 Infl.	0.5	22*	41**	172**	136*	11	15	18	13
	5	17*	44**	142**	176**	12	15	15	12
	25	19*	40**	138**	158**	17	15	11	10
	100	17*	41**	107**	165**	17	16	8	8
	500	13*	45**	191**	200**	15	16	11	10

BEGIN LAST LINE OF TEXT HERE
(continued)
BOTTOM OF IMAGE AREA, OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

TABLE 8 (continued)

Sample No.	Dose in µl	98 no S9	98 S9	100 no S9	100 S9	1535 no S9	1535 S9	1537 no S9	1537 S9
20 Effl.	0.5	7	33	57	108	9	14	5	14
	5	10	44	92	115	8	6	8	8
	25	20	37	86	138	11	11	8	11
	100	12	37	114	139	11	14	8	12
	500	16	38	126	136	6	14	8	9
22 Infl.	0.5	15	30	79*	80	5	9	6	8
	5	17	26	69*	106	8	11	9	11
	25	14	43	75*	98	11	7	7	9
	100	22	32	98*	130	3	10	9	11
	500	22	28	92*	126	10	10	10	6
26 Infl.	0.5	20	29	123	139	14	8	7	12
	5	20	17	156	130	11	10	8	14
	25	18	25	129	137	12	10	6	3
	100	14	34	166*	202	12	11	5	14
	500	18	21	124*	178	8	16	8	15
30 Infl.	0.5	14*	38*	128*	187*	14	10	15	13
	5	16*	36*	173*	195*	12	12	7	9
	25	22*	39*	182*	203*	13	15*	12	10
	100	22*	42*	151*	201*	11	16	15	11
	500	22*	42*	167*	200*	11	20*	10	20
32 Effl.	0.5	11	30	115	137	9	25*	11	7
	5	11	37	123	149	16	44*	6	11
	25	13	26	135	147	13	13*	5	11
	100	11	28	165	139	8	12	59	30
	500	16	27	130	160	10	13	5	9
34 Infl.	0.5	9	21	63*	82*	5*	9	2	10
	5	14	20	81*	135*	8*	6	6	6
	25	24	31	103*	114*	10	9	8	13
	100	12	25	122*	101*	8	15	4	9
	500	5	54	121*	139*	12	17	6	15

BEGIN LAST LINE OF TEXT

(continued)

BOTTOM OF IMAGE AREA; OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE

TABLE 8 (continued)

Sample No.	Dose in µl	98 no S9	98 S9	100 no S9	100 S9	1535 no S9	1535 S9	1537 no S9	1537 S9
36 Efl.	0.5	23	37	131	144	10	14	12	8
	5	17	32	117	118	14	11	11	9
	25	23	31	140	123	18	8	10	12
	100	20	41	119	142	13	14	12	15
	500	18	37	130	118	19	10	9	10
38 Infl.	0.5	16	27	165*	181*	8	12	10	10
	5	23	34	163**	173*	8	15	9	8
	25	9	44	192**	201**	11	13	6	9
	100	15	35	167**	184	16	12	6	10
	500	30	27	184**	148	7	12	15	13
38 Infl. C-T	500					25			
40 Efl.	0.5	18*	16	150*	80*	5	4	7	8
	5	13	22	151*	75*	2	6	6	4
	25	17	32	82*	101*	10	7	5	13
	100	18	31	82*	108*	11	15	9	8
	500	17	24	102*	96*	20**	22**	9	9
42 Infl.	0.5	5	34	177**	96*	6	8	10	12
	5	7	36	186**	169*	15	10	7	10
	25	9	46	205**	162*	14	13	8	14
	100	9	39	199**	173**	8	15	12	17
	500	6	35	203**	166*	9	15	7	8
48 Efl.	0.5	31	45	93	128	9	5	20	9
	5	16	35	97	115	12	10	6	12
	25	16	36	95	125	8	10	8	8
	100	24	31	87	132	8	9	3	11
	500	17	34	106	111	7	8	7	12

9-1/8"
BEGIN LAST LINE OF TEXT
(continued)
BOTTOM OF IMAGE AREA. OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"

EPA-287 (Cin.)
(4-76)

BEGIN FIRST LINE OF TEXT HERE
TYPING GUIDE SHEET
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE
6-1/2"
9-1/8"
BEGIN LAST LINE OF TEXT
3/8"
BOTTOM OF IMAGE AREA; OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
PAGE NUMBER
EPA-287 (Cin.) (4-76)

TABLE 8 (continued)

Sample No.	Dose in µl	98		100		1535		1537	
		no S9	S9	no S9	S9	no S9	S9	no S9	S9
50 Infl.	0.5	26	45	169*	176**	18	16*	11	12
	5	27	38	185*	205*	13	6	6	11
	25	25	33	153*	164*	12	11	13	11
	100	43	33	173*	158**	10	14	13	4
	500	50	46	254**	221**	15	15	10	12
54 Effl.	0.5	10	41	105	144	11	10	6	12
	5	11	32	122	155	13	15	8	8
	25	14	32	135	156	13	13	8	9
	100	11	32	122	169	10	8	8	11
	500	10	32	117	170	13	14	8	7